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CONTROL OF THERMOPHILIC SPORE ACTIVITY WITH PRESSURIZED CO_2 AND EGG-WHITE LYSOZYME

by

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The shelf life of low-acid (pH 5.4-5.6), thermally processed military rations, such as mixed vegetables or stews, can be limited by the metabolic activity of gram-positive, heat-resistant spore-formers, such as Bacillus stearothermophilus. For that reason, an investigation was initiated to evaluate the feasibility of using carbon dioxide (CO_2) alone or in combination with egg white lysozyme to inactivate foodborne thermophilic sporeformers. Results showed that vegetative cells of B. stearothermophilus were very sensitive to hyperbaric (pressurized) CO_2. During 3.5 h of incubation in AAMS broth at 25^oC and 200 psi CO_2, a 5-6 $\log_{10}$ cycle decrease occurred in the cell density. Similarly, at 400 psi CO_2, a 6-7 log decrease occurred in the cell population. However, in the spore state, results indicated that B. stearothermophilus was more resistant to CO_2 inhibition than vegetative cells. It was shown that when heat-resistant spores of B. stearothermophilus (10^3-10^4 spores/mL) were cultured in AAMS broth treated with CO_2 (0 and 400 psi) and egg white lysozyme (0,150 and 300 ppm) for up to 24 h at 37^oC, inhibition occurred. In the presence of 400 psi CO_2, samples treated with 150 ppm lysozyme averaged a 1.42 log decrease in spore density over 24 h incubation (37^oC), while 300 ppm lysozyme resulted in an average spore reduction of 4.5 logs, under similar storage conditions. In the absence of CO_2, enzyme-treated samples, 150 and 300 ppm, averaged a 0.91 and 3.36 log spore reduction during 24 h storage, respectively. Indications are that pressurized CO_2 may act synergistically with lysozyme.

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SHELF LIFE, MILITARY RATIONS, LYSOZYME, BACILLUS STEAROTHERMOPHILUS, FOODBORNE VIRUS, VEGETATIVE CELLS, METABOLIC ACTIVITY, LYTIC ENZYMES, CARBON DIOXIDE, INACTIVATION, SPORES

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PREFACE

The research described herein analyzes the effects of a combination treatment of hyperbaric CO_2 and lytic enzyme (egg white lysozyme) on the inactivation of heat-resistant, bacterial endospores (Bacillus stearothermophilus). Preliminary results indicated that under low CO_2 pressure, e.g., $\leq$500 psi, vegetative cells of B. stearothermophilus tended to be highly sensitive to pressurized CO_2; however, pressurized CO_2 in the range of 800-1100 psi had no apparent effect on the viability of B. stearothermophilus spores for as long as 96 hours of storage. Preliminary results also indicated in the presence of a lytic enzyme, such as egg white lysozyme, spore destruction could be achieved. In the presence of pressurized CO_2 (400 psi) and lysozyme (300 ppm), the results indicated that the rate of spore destruction was more rapid. Thus the focus of this research is on the relationship between pressurize CO_2 and lysozyme and its impact on spore viability.

This work, which began on 30 October, 1990 and ended 30 September, 1993, was supported under the project entitled "Hyperbaric Preservation," AH5240DOO.

CONTROL OF THERMOPHILIC SPORE ACTIVITY WITH PRESSURIZED CO_2 AND EGG-WHITE LYSOZYME

INTRODUCTION

Carbon dioxide (CO_2) has been shown to inhibit the metabolic activity of vegetative bacteria. This fact has been exploited in preventing bacterial food spoilage through modified atmosphere packaging (MAP). The ability of high concentrations of CO_2 to retard the growth of spoilage flora in red meat, poultry and fish is well documented (1,2,10,11,14).

The inhibitory effects of CO_2 increase when it is applied under pressure (2,5,6,8). Hyperbaric carbon dioxide has been shown to be effective against gram-positive thermophilic sporeformers (6). Egg white lysozyme has also been shown to have antibacterial properties (9). It is especially effective against gram-positive bacteria, such as *Bacillus stearothermophilus* (4). The current investigation looks at the antibacterial activity of the individual and combined effects of CO_2 and lysozyme against a foodborne, thermophilic sporeformer. Thus the objectives of the present investigation were to determine the effects of hyperbaric CO_2 alone and in combination with egg white lysozyme on the viability of vegetative cells and spores of *B. stearothermophilus*.

MATERIALS AND METHODS

Spore preparation. A stock culture of *Bacillus stearothermophilus* spores (ATCC 12980) was obtained from the culture collection of the Microbiology Section, U.S. Army RD&E Center, Natick, MA. Cultures were maintained on Cook and Brown sporulation agar slants (3), stored at 1-4°C and transferred monthly to maintain active stock cultures. Before spores were prepared, stock cultures were activated by suspending a loopful of the stock in phosphate buffer, pH 6.8, surface plating on antibiotic assay agar + 0.1% soluble starch (AAMS) and incubating for 24 h at 55°C. Using phosphate buffer (0.05M potassium phosphate, pH 6.8), the sporulation inoculum was prepared by scraping (bent glass rod) the 24 h growth from the AAMS plates and adding 3 mL volumes to Fernbach flasks.

All spores used in this study were prepared on Cook and Brown sporulation agar (3) according to the procedures described by Feeherry et al. (7). After incubating for 4 d at 55°C, spores were harvested from Fernbach flasks and were washed 3x with phosphate buffer (pH 6.8). Subsequently, the pellets were suspended in 200 mL of containing 100 micrograms/mL of lysozyme and incubated with stirring at 37°C for 1 h. Enzyme-treated spores were washed 4x with cold, distilled water to remove vegetative debris. Pellets were resuspended in cold, sterile phosphate buffer and stored at ~4°C until used.

Spore _activation_. Prior to use in an experiment, the spore inoculum (10^6 spores/mL) was heat activated by subjecting to flowing steam for 10 minutes.

Lysozyme _preparation_. Egg-white lysozyme and _Micrococcus luteus_ (ATCC 4698) were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. It was determined that the enzyme preparation contained approximately 34,600 units/mg. One unit of enzyme caused a decrease in turbidity at 540 nm of 0.0009/minute at pH 6.7 (13).

Hyperbaric _treatment_. Vegetative cells or spores were suspended in either sterile 0.05M phosphate buffer (pH 6.8), 0.05M acetate buffer (pH 4.5), 0.05M acetate buffer (pH 4.0) or AAMS broth (pH 6.8) in 16 x 150 mm screw-capped or 25 x 150 mm culture tubes. The caps were loosely secured to permit free gas exchange. The tubes were placed in the cylinder of Parr cell disruption bombs (Parr Instrument Company, Moline, IL; capacity: 1850 mL). After loading, the cylinders were sparged with either nitrogen or carbon dioxide at 200 psi for one minute to remove air. The cylinders were then pressurized (50-1100 psi) and stored at various temperatures (3°, 25°, 37°, 55°, 65°, 90° and 100°C) for 1 to 96 h.

Bacterial _enumerations_. Vegetative cells or spores were enumerated by spread plating (0.1 mL) on AAMS agar at 55°C. Serial dilutions were prepared with either sterile deionized water or 0.05M phosphate buffer, pH 6.8. All counts reported represent the mean of two replicates with duplicate

samples/replicate (n = 4).

RESULTS

Effect on vegetative cells. Hyperbaric CO_2 has been shown to have a bactericidal effect on vegetative cells of *B. stearothermophilus* (12). Destruction of vegetative cells was shown to be dependent on both time of exposure and CO_2 pressure.

The effect of 200 psi CO_2 at 25°C on survival of vegetative cells over a 3.5 h period is shown in Fig. 1. The cell population decreased over 4 log cycles (log 6.3 to > log 2.0) during the 3.5 h treatment period at 25°C. The data in Fig. 2 suggest that 400 psi achieved sterilization treatment when a population of vegetative cells was exposed for 1.5 h at 25°C. Nitrogen gas at the same pressure had no adverse effect on viability of *B. stearothermophilus* vegetative cells (12).

Effect on endospores. Roskey and Sikes (12) found that heat-resistant endospores of *B. stearothermophilus* were extremely resistant to the bactericidal effects of hyperbaric CO_2 at 55°C. The effect of CO_2 and N_2 on the survival of *B. stearothermophilus* spores under varying conditions of temperature and pH is shown in Table 1. When spores (~log 4.7 spores/mL) were suspended in sterile 0.05M acetate buffer (pH 4.0), 0.05M acetate buffer (pH 4.5) or 0.05M phosphate buffer(pH 6 .8) and then subjected to hyperbaric CO_2 (540-1100 psi) for 1 to 96 h, there was no apparent spore destruction, except at 90°C and pH 4.5.

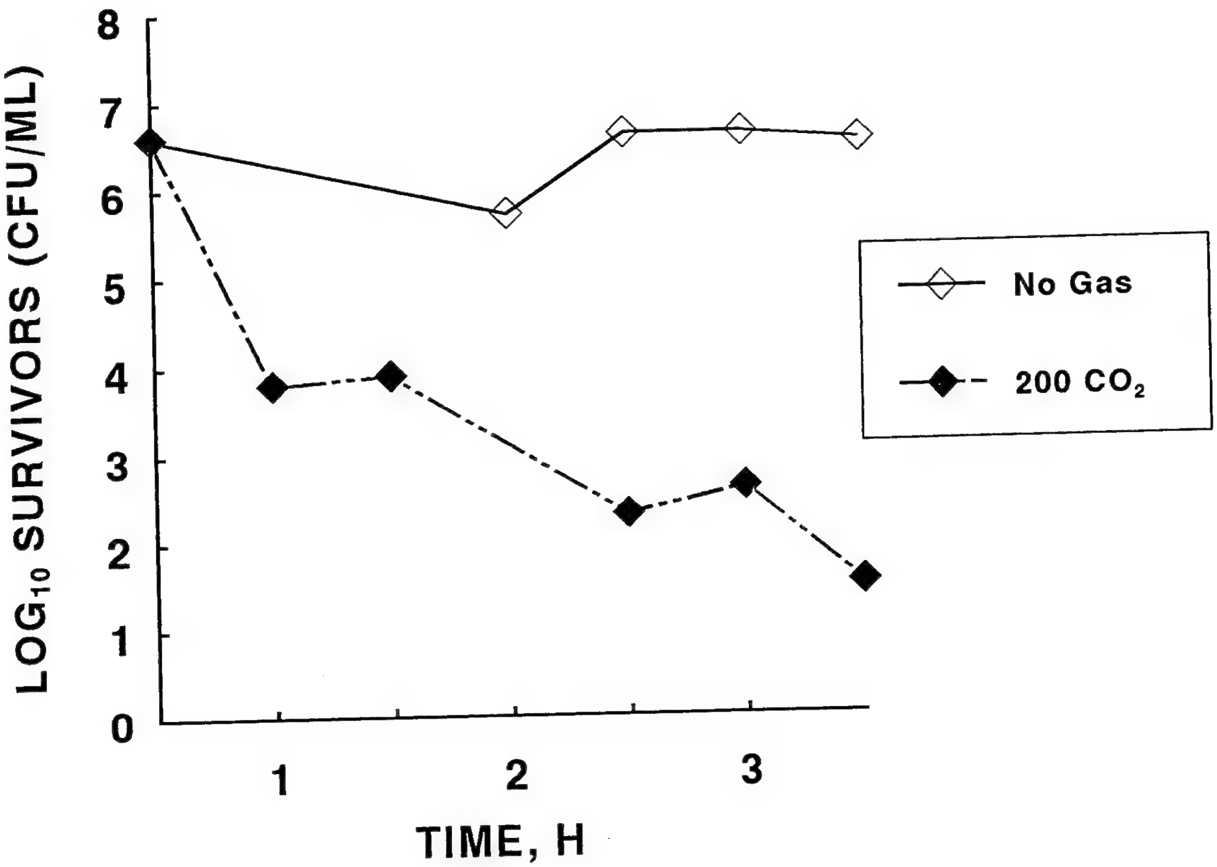

Figure 1. Survival of vegetative cells of B. stearothermophilus when exposed to 200 psi CO_2 for 3.5 h in AAMS broth (pH 6.8) at 25°C and enumerated on AAMS agar at 55°C. Data points are mean values, n = 4 (12).

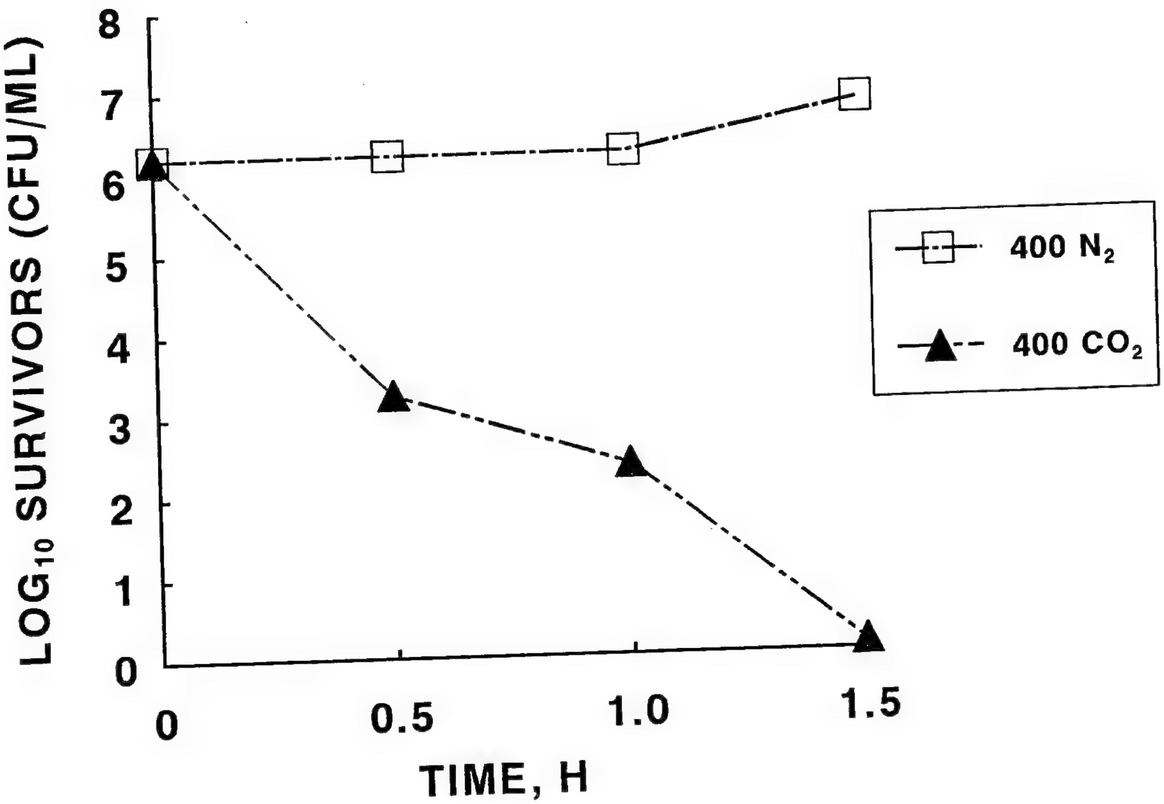

Figure 2. Survival of vegetative cells of B. stearothermophilus when exposed to 400 psi CO_2 or N_2 gas for 1.5 h in AAMS broth (pH 6.8) at 25°C and enumerated on AAMS agar at 55°C. Data points are mean values, n = 4 (12).

Table 1. Effects of hyperbaric carbon dioxide on the viability of <u>Bacillus</u> <u>stearothermophilus</u> spores (12).

pH	Temp. °C	Pressure psi	Time hrs	Survival Fraction log $^{b}N_{CO2}/^{a}N_0$	log $^{c}N_A/N_0$	log $^{d}N_{N2}/N_0$
4	3	800-550	68	0.98	0.98	________
4	25	840-890	68	0.94	0.93	________
4	55	840-1050	68	0.99	0.87	________
4	65	800-900	45	0.98	0.86	0.82
4.5	3	820-540	96	1.04	1.06	1.06
4.5	25	830-850	96	1.01	.98	________
4.5	65	840-900	23	0.99	.82	0.89
4.5	90	850-875	22	0.55	.78	________
7	25	800-930	23	1.01	0.96	1.01
7	55	940-1050	46	1.01	0.80	0.81
7	65	940-975	20	1.00	0.81	________
7	75	875-1000	26	1.06	0.91	________
7	100	900-1100	1	1.09	1.08	________

$^{a}N_0$ = initial population (mean initial population was $\log_{10}$ 4.7)

$^{b}N_{CO2}$ = number present after CO_2 treatment.

$^{c}N_A$ = number present after exposure to ambient gas atmosphere for the treatment period.

$^{d}N_{N2}$ = number present after exposure to N_2 gas at the same pressure as CO_2 for the same time.

Aliquots of the same spore suspensions subjected to ambient gas conditions and those subjected to hyperbaric N_2 were similarly unaffected.

Examination of the data in Table 1 also showed that spores exposed to high temperatures (90°C) and low pH may act synergistically with hyperbaric CO_2 (850-875 psi). Additional studies will be necessary to characterize more clearly the synergistic effects of high temperature, low pH and hyperbaric CO_2.

Effect of lysozyme on endospores. When Bacillus stearothermophilus spores were incubated in AAMS broth containing 300 ppm egg-white lysozyme in the absence of pressurized CO_2 and, subsequently, plated on AAMS agar, the spore count was reduced to <1 CFU/mL, between 2 and 4 h incubation at 37°C (Fig. 3). Under similar experimental conditions, 150 ppm lysozyme had no apparent effect on spore viability during a 24 h incubation period (Fig. 3). Thus, it would appear that at 150 ppm lysozyme spore viability is unaffected, but spore germination/outgrowth was inhibited.

In the absence of enzyme treatment, B. stearothermophilus spores germinated and grew out to a density of $\log_{10}$ 6-7 CFU/mL, during 24 h incubation (37°C) in AAMS broth (Fig. 3).

In the presence of 200 psi hyperbaric CO_2 and the absence of lysozyme, a slight increase (< 1 $\log_{10}$) occurred in the CFU/mL during the initial 2-4 h incubation in AAMS broth at

37°C (Fig. 4). Subsequently, spore numbers remained unchanged (Fig. 3).

In the presence of 150 ppm lysozyme and 200 psi CO_2, a similar pattern of growth and dormancy was observed (Fig 3). In the presence of 300 ppm lysozyme and 200 psi CO_2, spore viability was reduced to < 1 CFU/mL in less than 2 h incubation in AAMS broth (37°C; Fig. 4) .

Similarly, at a CO_2 pressure of 400 psi and lysozyme concentrations of 150 and 300 ppm, the CFU/mL increased slightly (< 1 $\log_{10}$) during the initial 2 h of incubation in AAMS broth (Fig. 5). Subsequently, the viable spore count remained essentially unchanged during the next 6 h of incubation but decreased slightly between 8 and 24 h (<1 log; Fig. 5). At an enzyme concentration of 300 ppm, the viability spore count was immediately and dramatically affected. After 2 h of incubation in AAMS broth, the viable spore count decreased to < 1 CFU/mL for a period of 22 h (Fig. 5).

It was also apparent from the data in Figures 3-5 that in the absence of enzyme treatment the viable spore count always was higher than any of the enzyme treated samples, even in the presence of CO_2 pressure.

Thus, at the treatment levels used in this study, the combination treatments (enzyme + pressure) may indicate that the combination treatments are more effective than individual treatments; however, more definitive data are required before this conclusion can be reached.

pressure CO_2 treatment (11).

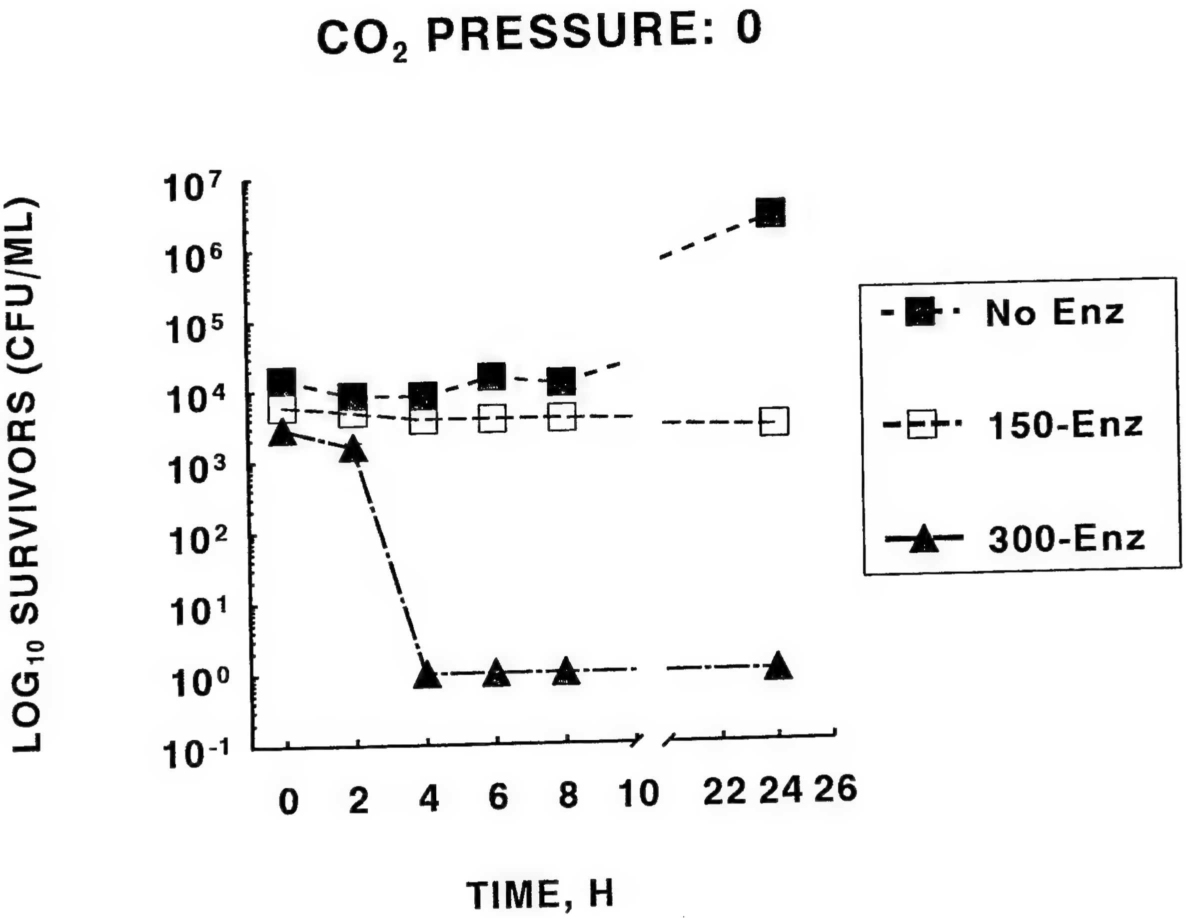

Figure 3. Effect of egg-white lysozyme (150 and 300 ppm) on the viability of B. stearothermophilus spores incubated in AAMS broth (pH 6.8) at 37°C and enumerated on AAMS agar at 55°C. Data points represent mean values of two replicates with two samples/replicate (n = 4).

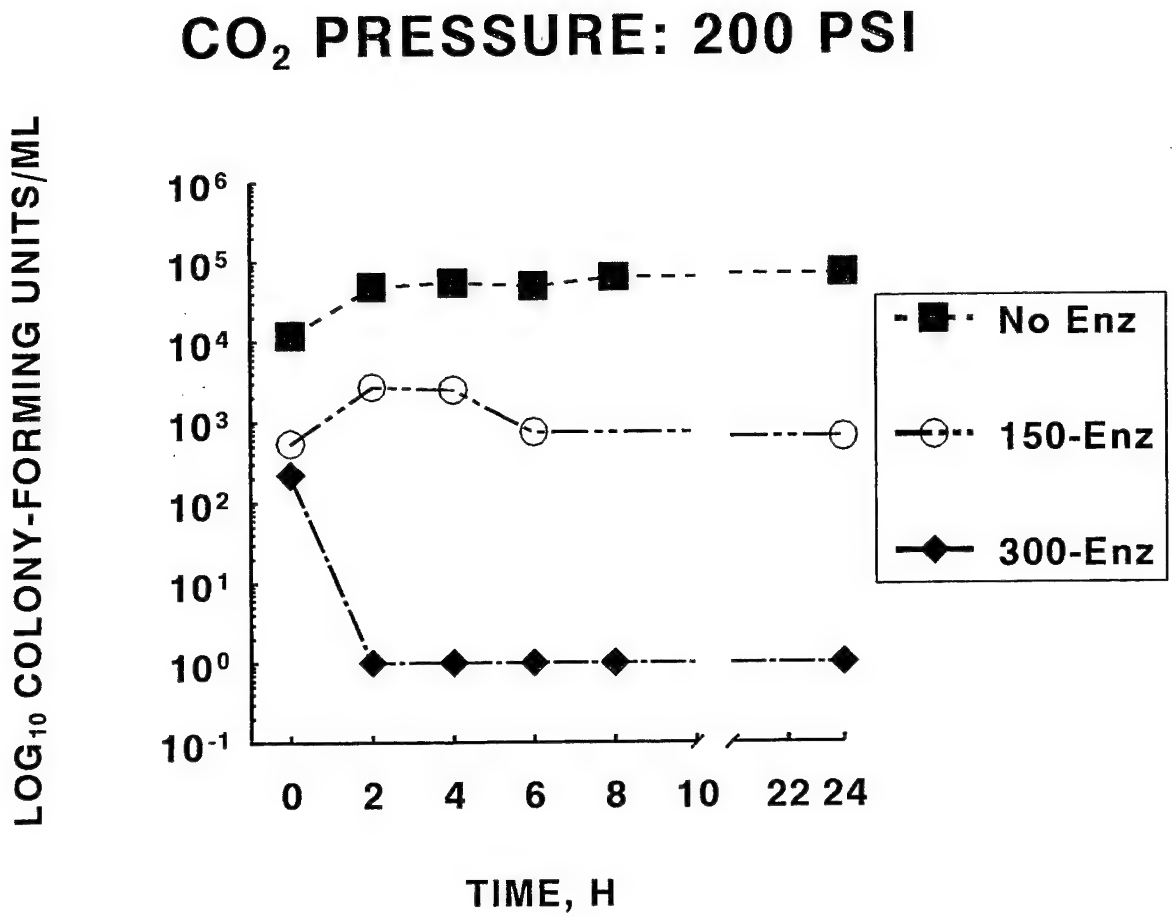

Figure 4. Combined effects of egg-white lysozyme (150 and 300 ppm) and pressurized CO_2 (200 psi) on the viability of B. stearothermophilus spores incubated in AAMS broth (pH 6.8) at 37°C and enumerated on AAMS agar at 55°C. Data points represent the mean values of two replicate experiments with two samples/replicate (n = 4).

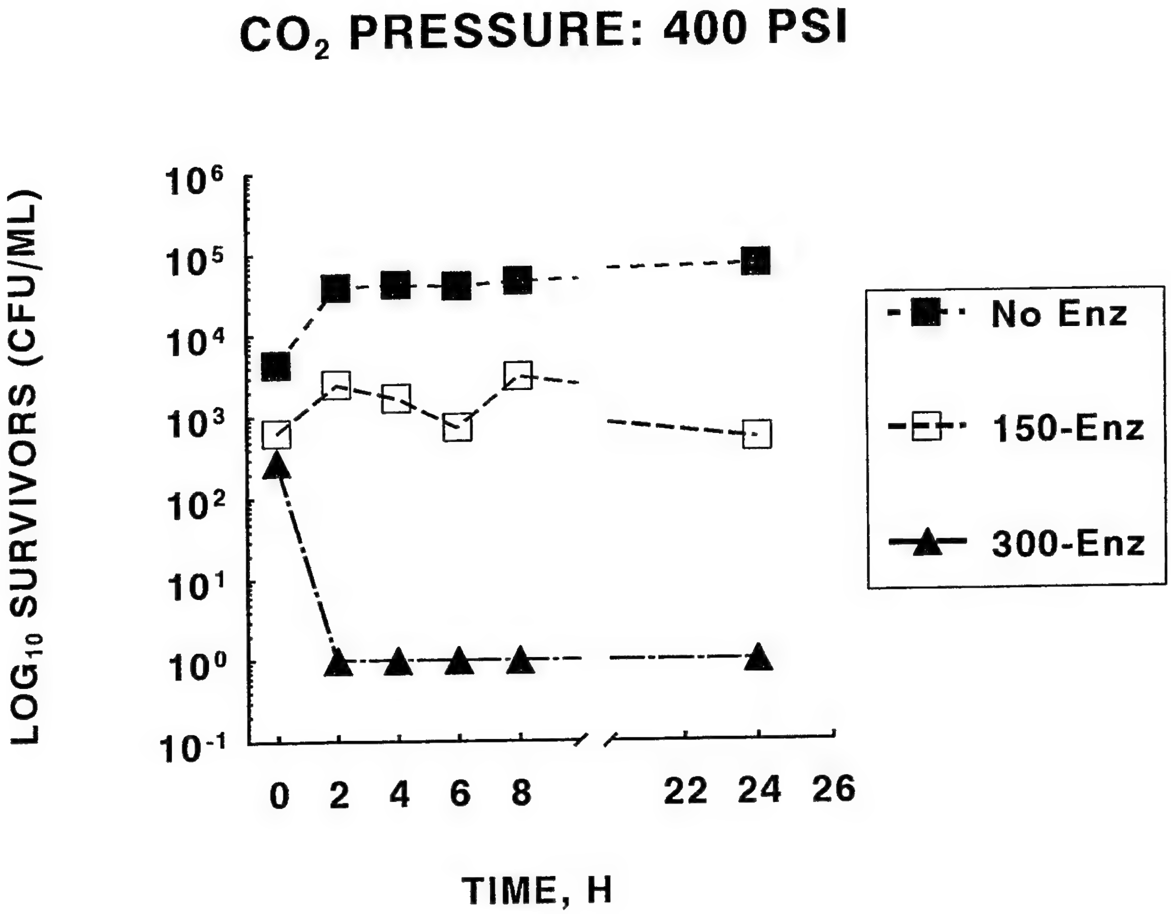

Figure 5. Combined effects of egg-white lysozyme (150 and 300 ppm) and pressurized CO_2 (400 psi) on the viability of B. stearothermophilus spores incubated in AAMS broth (pH 6.8) at 37°C and enumerated on AAMS agar at 55°C. Data points represent the mean values of two replicate experiments with two samples/replicate (n = 4).

SUMMARY AND CONCLUSIONS

Vegetative cells of B. stearothermophilus were found to be extremely sensitive to relatively low levels of pressurized CO_2. Complete destruction (no recovery at 55°C) of 10^6 vegetative cells/mL in AAMS broth was obtained by exposure to 400 psi CO_2 for 1.5 h (no survivors).

Spores, however, were more resistant and remained viable after exposure to 800-1100 psi CO_2 for as long as 96 h. Spores also survived CO_2 treatment at low (3°C) as well as high (100°C) temperatures and at pH 4, 4.5 and 7.0. However, the data obtained suggested that high pressure CO_2 (850-875 psi) exposure at high temperature (90°C) may act synergistically to compromise spore viability. In the absence of CO_2 (37°C), no apparent difference was evident between spore viability in the presence or absence of 150 ppm of lysozyme; however, at an enzyme concentration of 300 ppm (no CO_2), spore viability was reduced to < 1 CFU/mL in < 4 h of incubation.

In the presence of CO_2 pressure (200 and 400 psi), there was a difference in spore viability in the presence or absence of enzyme. Under pressure, a 1-2 $\log_{10}$ differential existed between the control (no enzyme treatment) and the 150 ppm lysozyme treatment. Pressurized CO_2 also resulted in a more rapid spore destruction rate (<2 h).

Since the rate of spore destruction was apparently more rapid in the presence of pressurized CO_2, pressurized CO_2 and

lysozyme may act synergistically against *B. stearothermophilus* spores. To confirm this observation, additional research will be required.

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